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Accumulation of Lysophosphatidylcholine and Lysophosphatidylethanolamine in Muscle of Fresh Skipjack

by Toshiaki OHSHIMA* and Chiaki KOIZUMI*

(received November 17, 1982)

Phospholipids of the muscle of fresh skipjack *Euthynnus pelamis* were analyzed by HPLC, GLC, and TLC. The contents of lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) in skipjack muscle were extremely high, though earlier workers had reported that the contents of LPC and LPE produced enzymatically in the fish muscle were considerably low and did not increase during frozen storage. The accumulation of LPC and LPE in the skipjack muscle might be elucidated by either saying that the activity of phospholipase A_1 is higher than that of lysophospholipase or that the lower pH of skipjack muscle influences the activity of lysophospholipase; the optimum pH of lysophospholipase in fish muscle is known to be around 7.6.

The fatty acid compositions of phosphatidylcholine (PC), LPC, and free fatty acid were analyzed. Percentage of $C_{22:6}$ was considerably high in the PC, while extremely low in the LPC. These results seem to indicate either that most of the PC having $C_{22:6}$ located on the α-position of glycerol is not attacked by the action of phospholipase A_2 or that the LPC having $C_{22:6}$, if produced from PC by the action of phospholipase A_2, is attacked preferentially by the action of lysophospholipase to librate $C_{22:6}$.

The possible fatty acid combinations of the PC remaining in the skipjack muscle were also presented.

During frozen storage, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in fish meat are hydrolyzed into lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) through the action of phospholipase A_2 (EC 3.1.1.4). Furthermore, these lyso-type phospholipids are said to be hydrolyzed into free fatty acids (FFA) and glycerophosphatidylcholine or glycerophosphatidylethanolamine through the action of lysophospholipase (EC 3.1.1.5)[1]. The enzyme systems participating in the hydrolysis of these phospholipids are originally present in the flesh of the fish and are said not to originate from microorganisms.[2-5] So, one may expect

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not only PC and PE, but also LPC and LPE to be present in the flesh of fish kept in frozen storage. On the basis of the research conducted so far, it was said that there was no LPC or LPE, or that if there was any their volume was very small and that they were virtually not increasing during frozen storage.[6-10] The reason given was that the activity of the lysophospholipase present in the flesh of the fish was remarkably higher than that of phospholipase.[7,8]

However, Braddock and Dugan Jr.[11] reported that although LPC in the flesh of coho salmon decreases during frozen storage, LPE increases. It is known that the lyso-type phospholipids accumulate in poultry as the phospholipids get hydrolyzed[12], but there had been no report on lyso-type phospholipid accumulation in fish other than coho salmon.

We wanted to examine in detail the changes in fish meat phospholipids during frozen storage by new methods using high-speed liquid chromatography (HPLC). As we made analyses, choosing fresh skipjack samples, we saw that large amounts of LPC and LPE accumulated in the flesh of the fish. We present here our results.

Experimental

Sample fish - In June 1982, we purchased from retail shops fresh skipjack Euthynnus pelamis of 1.8 kg which we used as samples.

Extraction and fractionation of the lipids - We minced the ordinary muscle of the samples and extracted the total lipids (TL) by the Bligh and Dyer method[13], using $CHCl_3$-MeOH. To fractionate the TL into polar lipid portion (PL) and nonpolar lipid portion (NL), we relied on column chromatography[15] using biobeads S-X2 as in our former report. p. 1206

HPLC of PL - We used 2 Partisil 10 SCX columns with different inner diameters. We performed HPLC using a column with an internal diameter of 4.6 mm in the same manner as in our former report[14]. As for the HPLC using a column with the inner diameter of 9.4 mm the elution rate was set at 5.8 mL/min. The other conditions were similar.

Densitometry - The silica gel thin-layer plate densitometry was conducted as in our former report[14].

Infrared absorption spectrometry - The IR absorption spectra were measured with a JASCO IRA-2 spectrometer. The samples were made into a chloroform solution, placed on a KCl plate and a solvent was vaporized.

PC fatty acid combination estimation method - We operated as in our former report[14].

Standard LPC and LPE - We used eggyolk LPC and LPE from the Sigma Co.

Results and discussion

Lipid contents and fatty acid composition - The amount of lipids in the sample skipjack meat is 1.09 g/100 g of which PL represents 0.58 g/100 g and TL 53%.

The fatty acids compositions are shown in Table 1. With regard to the TL fatty acid composition, the composition ratio of the highly unsaturated fatty acid $C_{22:6}$ is highest, followed by $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, in this order. The NL fatty acid composition resembles that of TL. Yet the highest fatty acid composition ratio was held by $C_{16:0}$.

Meanwhile, the PL fatty acid composition differed markedly from the 2 above. $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{22:6}$ and the like dominated. Of these, $C_{22:6}$ held a very high composition ratio, representing 42%. The TL fatty acid compositions shown here agree well with the results reported by Roubal[16]. p. 1207

PL separation in HPLC - Fig. 1 shows the HPLC chromatogram obtained with a column having a 4.6 mm inner diameter. In the PL of cod meat presented in a former report[14], only 2 peaks appeared, but 4 peaks up to P_1~P_4 appeared in the PL of skipjack meat. Under the HPLC conditions adopted in this study, phosphatidylserine is contained in the first solvent peak. It does not appear as an independent peak. From the retention time (Rt) of the standards, P_1 was judged to be PE, P_2 LPE, P_3 PC and P_4 LPC. We gathered the components of the various peaks for confirmation.

For gathering the fractions, we used a column with an inner diameter of 9.4 mm. The obtained chromatograms are shown in Fig. 2. There is some difference from the chromatogram seen in Fig. 1. The portion marked Fr. 1 in Fig. 2 contains P_1 and P_2 in Fig. 1. Fr. 2 contains P_3, and Fr. 3 contains P_4. By repeating the HPLC, we obtained the 3 fractions Fr. 1 - Fr. 3.

Identification of phospholipids by TLC - We analyzed the gathered Fr. 1 - Fr. 3 by the two-dimensional thin layer chromatography (TLC)[17] used in the former report[14]. As a result, Fr. 2 showed a single spot and Fr. 1 and Fr. 3 showed 2 spots each. From the Rf values we estimated that Fr. 1 consisted of PE and LPE, that Fr. 2 consisted of

PC, and Fr. 3 of sphingomyelin (SPM) and LPC. So, we submitted Fr. 1 to TLC with $CHCl_3/MeOH/H_2O$ (65/25/4, v/v)[18] as development solvent, using silica gel G. We gathered separately the 2 spots appearing at Rf 0.63 (C_1) and Rf 0.20 (C_2) and extracted with petroleum ether/ether/ $MeOH/CHCl_3$ (4/4/1/1, v/v)[19]. TLC was similarly performed on Fr. 3 and we obtained the 2 fractions C_3 (Rf 0.11) and C_4 (Rf 0.17). p. 1208

We performed TLC again on the 4 fractions $C_1 \sim C_4$ thus obtained and on the single spot P_3 yielded by TLC. We compared with the Rf values of standard products and performed the color reaction on a thin-layer plate. The results are shown in Table 2. As all fractions are positive to Molybdenum blue reagent[20], they contain phosphorus. As P_3, C_3 and C_4 are positive to the Dragendorff reagent[21], they contain choline. Furthermore, as C_1 and C_2 are positive to ninhydrin reagent[22], they contain amino groups. From these results and from comparing with the Rf values in TLC and with the Rt standards in HPLC, P_1 in Fig. 1 was judged to be PE, P_2 LPE, P_3 PC, P_4 LPC and the small shoulder just ahead of P_4 was judged to be SPM. The LPC fraction was further measured by IR absorption spectrum. The results are shown in Fig. 3. The 1087 and 1053 cm^{-1} absorptions are particular to choline, the 962 and 1225 cm^{-1} absorptions are particular to phosphoric acid and the 1465 cm^{-1} absorption is particular to the methyl group. They agree well with the spectra of the standards.

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Composition of the PL and NL components - We submitted PL to TLC and computed by densitometry the composition ratios of the various phospholipids. Table 3 shows the composition of the PL components in 100 g of fish meat calculated from the above and from 0.58 g/100 g which is the content in fish meat. The table shows a marked LPC

accumulation. However, as LPE is not completely separated from PC under the present TLC conditions, the amount of accumulation is unclear. The estimation based on the PE in Fig. 1 and on the specific area of the peaks indicates about 21.1 mg/100 g which is fairly lower than LPC.

Similar calculations were made in Table 4 showing the composition of the NL components. The FFA contents are highest. Olley et al[23] state that lipase and phospholipase play an important role in the FFA increase during low-temperature storage of fish. It is also said that up to 25% of the FFA accumulated in the meat of Baltic herring[10] originates from triglyceride (TG). There are also reports that all FFA accumulated in fish meat during frozen storage originates from phospholipids.[24] However, Tsukuda[19] estimates that the FFA increase during frozen storage of skipjack is not due solely to phospholipids but partly to TG as TG goes on decreasing. In this study also, the low TG contents seem to indicate that part of the FFA originates from the hydrolysis of TG.

As shown above, much LPC, LPE and FFA was accumulated in the meat of fresh commercial skipjack. Later, we analyzed separately frozen skipjack and found indeed practically the same LPC, LPE accumulations. It is thus certain that skipjack meat is prone to lysotype phospholipid accumulation and this may be a common characteristic of red fish exemplified in skipjack. We suspect that the reason why no lysotype phospholipid accumulation was noted in frozen-stored fish meat in the studies conducted so far is that many of the specimens used were white-meat fish.

The reason for lysotype phospholipid accumulation in skipjack meat is unclear. Is it that unlike in cod, the phospholipase activity is stronger than the lysophospholipase activity or is it that as skipjack meat has a low pH[25], this affects the activities of phospholipase and lysophospholipase? These are topics for further studies.

Composition of PC, LPC and FFA fatty acids

Table 5 shows the composition of the PC, LPC and FFA fatty acids. In the PC fatty acid composition, the composition ratio of $C_{22:6}$ is highest followed by $C_{16:0}$, $C_{18:1}$ in this order. As for LPC, $C_{16:0}$, $C_{18:1}$ and $C_{18:0}$ have high composition ratios. We saw no highly unsaturated fatty acid $C_{20:5}$ and the composition ratio of $C_{22:6}$ was markedly low. Regarding PC, the composition ratio of $C_{22:6}$ was remarkably high and it is noteworthy that $C_{22:6}$ was extremely low in LPC. According to Menzel and Olcott[26], the highly unsaturated fatty acids in the PC of salmon, menhaden and tuna meat are distributed from the α-position to the β-position and much $C_{16:0}$ is distributed at the α-position. The distribution of fatty acids in the PC of the skipjack used in this study is unclear. However, if we accept a large distribution of highly unsaturated fatty acids at the β-position as for the above fish, one may conceive of the 2 following possibilities on account of the low composition ratio of the highly unsaturated fatty acids in LPC. 1) The PC regarding which highly unsaturated fatty acids are distributed at the α-position does not easily undergo the action of phospholipase, 2) the LPC which at the α-position has highly unsaturated fatty acids produced from PC, undergoes readily the action

of lysophospholipase and is easily hydrolyzed. Both the composition ratio of $C_{22:6}$ of the residual PC not having undergone hydrolysis in case 1) and the composition ratio of $C_{22:6}$ in FFA in case 2) are supposed to increase. We wish to clarify this point later by trial storage of extremely fresh skipjack in which no LPC or LPE is produced.

Combination of the PC fatty acids

Tables 6 and 7 show respectively the composition of the hydrogenated PC fatty acids and the PC composition based on the total carbon numbers (TC) of the acyl groups. The odd-number carbon fatty acids C_{15}, C_{17}, C_{19} and C_{21} are included. The composition ratio of C_{15} is particularly high. It is reported that small quantities of the C_{15} and C_{17} odd-number carbon fatty acids are contained in mammary cells[27], mouse muscle[28], sweetfish[29], albacore[30], yellowfin tuna[30] and so on. In the present study, the composition ratio of PC in skipjack was not high. It is surmised that the reason why the composition ratio of odd-number carbon fatty acids in skipjack PC is high is the effect of the enzymatic hydrolysis undergone during low-temperature storage.

In the PC composition based on TC shown in Table 7, the composition ratios of TC 38, 44, 40, 36, 42, 34 and so on are high. TC 37, 39, 35 and the like dominate the PC containing odd-number carbon fatty acids. Among these the composition ratio of TC 37 is highest.

Table 8 shows the estimated PC fatty acid combinations obtained as in the former report[14] from these fatty acid compositions and from the TC-based PC compositions. For the computation, we used a FACOM M 140 F. If we compare the analytical values and the computed

values we see that aside from some differences in the composition ratios of the odd-number carbon fatty acids such as C_{17}, C_{19} and C_{21}, both PC compositions based on the fatty acid compositions and on TC agree well. The results of Table 8 are arranged in Table 9. The compositions of fatty acids in which the composition ratios are 5% or higher are (C_{16}, C_{22}), (C_{22}, C_{22}), (C_{18}, C_{22}), (C_{15}, C_{22}), (C_{16}, C_{18}), (C_{20}, C_{22}), (C_{16}, C_{20}) and (C_{18}, C_{20}). In the fatty acid composition of PC shown in Table 5, 86.5% of the C_{16} fatty acid is represented by $C_{16:0}$. As for the fatty acid C_{22}, 91.8% is represented by $C_{22:6}$. This is why most of the (C_{16}, C_{22}) combination having the highest composition ratio is thought to be ($C_{16:0}$, $C_{22:6}$). (C_{22}, C_{22}) which holds the next highest composition ratio may without much risk of error be regarded as ($C_{22:6}$, $C_{22:6}$). Among the C_{18} fatty acids, the composition ratio of $C_{18:1}$ is 74.3% so that most of the (C_{18}, C_{22}) combination seems to be represented by ($C_{18:1}$, $C_{22:6}$). It is the same thing with the other fatty acids. (C_{15}, C_{22}) may be considered to be ($C_{15:0}$, $C_{22:6}$), and (C_{16}, C_{18}) may be regarded as ($C_{16:0}$, $C_{18:1}$). With regard to the C_{20} fatty acid, there are some differences. $C_{20:4}$ and $C_{20:5}$ dominate the fatty acids C_{20}, and the respective composition ratios are 36.4% and 61.2%. Therefore, the (C_{20}, C_{22}) combination comprising the C_{20} fatty acids is most probably made from both ($C_{20:4}$, $C_{22:6}$) and ($C_{20:5}$, $C_{22:6}$). The same may be said about the (C_{18}, C_{20}) combination.

p. 1211

As Bosund et al[10] stated about the enzymatic hydrolysis of the phospholipids of Baltic herring kept in frozen storage, if no specific fatty acid undergoes preferential hydrolysis, the PC fatty acid

combination shown in Table 9 may be said to be the fatty acid composition of PC in shipjack muscle. This question should be researched further. Moreover, the fatty acid combinations shown here do not take into consideration the combination positions. This also should be researched further.

I wish to thank Mr. Shogo YAMAZAKI who helped with the tests performed in this study.

Table 1. Fatty acid compositions of total lipid (TL), polar lipid (PL), and non-polar lipid (NL) in skipjack muscle

(wt%)

Fatty acid	TL	PL	NL
14:0	0.8	0.7	0.9
:1	tr	tr	tr
15:0	2.3	4.8	0.7
16:0	24.5	9.2	33.5
:1	1.7	1.0	2.3
:2	0.6	1.0	0.3
17:0	1.8	1.7	1.8
18:0	10.0	6.4	11.4
:1	9.6	12.6	7.0
:2	0.9	0.7	1.0
:3	0.9	0.6	0.8
20:0	0.1	0.3	0.4
:2	1.1	0.3	0.4
:4	3.1	5.8	2.9
:5	6.5	6.2	6.3
22:0	0.1	0.2	0.1
:4	0.8	1.0	1.3
:5	3.9	5.1	2.2
:6	30.8	42.2	25.4
23:0	0.5	0.3	1.3

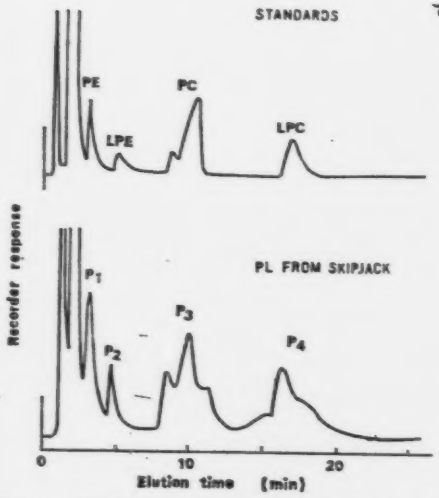

Fig. 1. HPLC chromatograms of the PL from skipjack muscle and standard mixture on Partisil 10 SCX column (4.6 mm i.d.).

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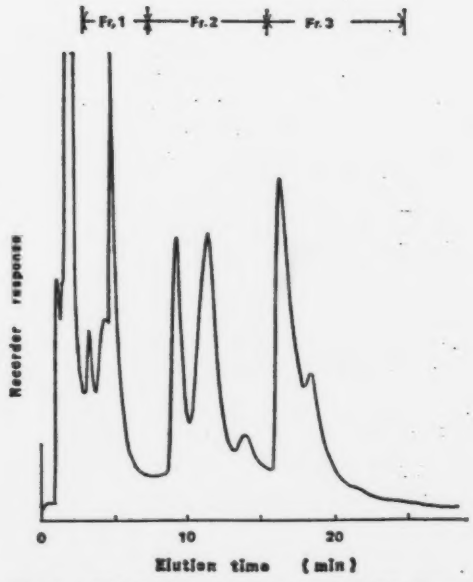

Fig. 2. HPLC chromatogram of the PL from skipjack muscle on Partisil 10 SCX column (9.4 mm i. d.).

Table 2. Rf values and color tests specific for phosphorus, choline, and amino residue

HPLC 1[*1]	HPLC 2[*2]	TLC	Rf		P	$N(\widetilde{CH_3})_3$	NH_2
			Solv. 1[*3]	Solv. 2[*4]			
P_1	Fr. 1	C_1	0.63	0.58	÷	−	+
		PE	0.62	0.57	÷	−	+
P_2		C_2	0.20	0.35	÷	−	+
		LPE	0.19	0.36	÷	−	+
P_3	Fr. 2	P_3	0.30	0.35	+	+	−
		PC	0.33	0.35	÷	+	−
P_4	Fr. 3	C_3	0.11	0.05	+	+	−
		LPC	0.10	0.05	÷	+	−
Shoulder		C_4	0.17	0.12	÷	+	−
		SPM	0.16	0.13	÷	+	−

*1 HPLC on Partisil 10 SCX column, 4.6 mm i.d. (Fig. 1)
*2 HPLC on Partisil 10 SCX column, 9.4 mm i.d. (Fig. 2)
*3 $CHCl_3/CH_3OH/H_2O$ (65/25/4)
*4 $CHCl_3/CH_3COCH_3/CH_3OH/CH_3COOH/H_2O$ (6/8/2/2/1)

Fig. 3

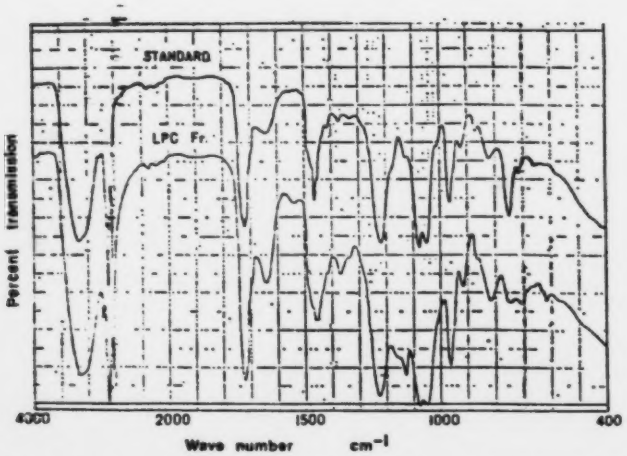

Fig. 3. Infrared spectra of the LPC from skipjack muscle and standard LPC.

Table 3. Phospholipid composition of the skipjack PL determined with densitometry

Lipid class*	Content (mg/100 g)
LPC	144
SPM	69
PC+LPE	226
PS	42
PE	98

* LPC, lysophosphatidylcholine; SPM, sphingomyelin; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; PS, phosphatidylserine; PE, phosphatidylethanolamine.

Table 4. Lipid composition of the skipjack NL determined with densitometry

Lipid class*	Content (mg/100 g)
SE+HC	31
TG	112
U. K.	7
FFA	213
ST, DG	145

* SE, sterol ester; HC, hydrocarbon; U. K., unknown; FFA, free fatty acid; ST, free sterol; DG, diglyceride.

Table 5. Fatty acid compositions of the PC, LPC, and FFA from skipjack muscle

(wt %)

Fatty acid	PC	LPC	FFA
14:0	0.4	—*	0.4
:1	tr	—*	0.1
15:0	5.8	—*	0.7
16:0	14.8	31.4	48.7
:1	1.5	9.6	0.9
:2	0.8	2.3	0.7
17:0	1.1	tr	2.2
18:0	2.7	12.5	18.0
:1	11.3	20.7	4.4
:2	0.6	tr	0.4
:3	0.6	0.5	0.3
20:0	0.2	4.8	0.2
:2	0.1	—	tr
:4	4.7	—	1.1
:5	7.9	—	3.1
21:0	—	0.9	3.2
22:0	0.2	8.2	—
:4	0.1	0.8	0.6
:5	3.6	3.8	1.1
:6	43.6	4.4	13.6
23:0	—	—	0.3

* Percentages of $C_{14:0}$, $C_{14:1}$ and $C_{15:0}$ were not able to be calculated due to interference of unknown peaks.

Table 6. Fatty acid composition of the PC after hydrogenation

Fatty acid	wt %	mol %
14:0	0.8	1.1
15:0	5.3	6.6
16:0	15.2	17.9
17:0	1.5	1.7
18:0	13.4	14.3
19:0	0.2	0.2
20:0	12.6	12.3
21:0	0.1	0.1
22:0	50.5	45.4
24:0	0.5	0.4

Table 7. PC composition based on the total carbon in fatty acyl chains after hydrogenation

TC*	wt %	mol %
32	1.4	1.6
33	1.3	1.5
34	7.5	8.2
35	3.0	3.2
36	10.2	10.7
37	8.1	8.4
38	26.0	26.3
39	4.0	4.0
40	11.1	10.8
41	1.4	1.3
42	7.8	7.3
43	1.5	1.4
44	16.8	15.2

* Total carbon in fatty acyl chains.

Table 8. Calculation of possible fatty acid combinations in the PC of skipjack muscle

Fatty acid composition	Total carbon in fatty acyl chains													(mol %)	
	C_{32}	C_{33}	C_{34}	C_{35}	C_{36}	C_{37}	C_{38}	C_{39}	C_{40}	C_{41}	C_{42}	C_{43}	C_{44}	Anal.*1	Cal.*2
C_{14}	0.09	0.04	0.10		1.50									1.1	1.7
C_{16}	0.20	0.67	0.05	1.63		4.34								6.6	6.9
C_{18}	0.51 0.51	0.04	3.90		3.06		10.40							17.9	18.4
C_{17}	0.20	0.04	0.05 0.05					2.06						1.7	2.4
C_{18}	0.09	0.67	3.90		0.78 0.78		2.85		5.41					14.3	14.5
C_{19}		0.04	0.05							0.72				0.2	0.8
C_{20}			0.10	1.63	3.06		2.85		0.14 0.14		3.91		0.10	12.3	11.9
C_{21}												0.70		0.1	0.7
C_{22}					1.50	4.34	10.40	2.06	5.41	0.72	3.91	0.70	7.91 7.91	45.4	44.9
C_{24}													0.10	0.4	0.1
Anal.*1	1.6	1.5	8.2	3.2	10.7	8.4	26.3	4.0	10.8	1.3	7.3	1.4	15.2		
Cal.*2	1.6	1.5	8.2	3.3	10.7	8.7	26.5	4.1	11.1	1.4	7.8	1.4	16.0		

*1 Analyzed
*2 Calculated

Table 9. Possible fatty acid combinations of the skipjack PC

TC	Combination of fatty acid	Content (mol%)
32	C_{14}, C_{18}	0.2
	C_{16}, C_{17}	0.4
	C_{16}, C_{16}	1.0
33	C_{14}, C_{19}	0.1
	C_{15}, C_{18}	1.3
	C_{16}, C_{17}	0.1
34	C_{14}, C_{20}	0.2
	C_{15}, C_{19}	0.1
	C_{16}, C_{18}	7.8
	C_{17}, C_{17}	0.1
35	C_{15}, C_{20}	3.3
36	C_{14}, C_{22}	3.0
	C_{16}, C_{20}	6.1
	C_{18}, C_{18}	1.6
37	C_{15}, C_{22}	8.7
38	C_{16}, C_{22}	20.8
	C_{18}, C_{20}	5.7
39	C_{17}, C_{22}	4.1
40	C_{18}, C_{22}	10.8
	C_{20}, C_{20}	0.3
41	C_{19}, C_{22}	1.4
42	C_{20}, C_{22}	7.8
43	C_{21}, C_{22}	1.4
44	C_{20}, C_{24}	0.2
	C_{22}, C_{22}	15.8

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